Regulatory T cell levels in children with asthma

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Natural regulatory T (nTreg) cells are described by expression of a specific transcription factor, FOXP3, on CD4+CD25+ cells. They play very important roles in the suppression of allergic reactions and disorders. The aim of this study was to obtain peripheral blood Treg levels among atopic asthmatic patients before and during inhaled steroid treatment and to observe the effect of these cells on the pathogenesis and treatment of asthma. CD4+CD25+FOXP3+ T cells obtained from 20 healthy donors and from 16 atopic asthmatic patients before and after inhaled glucocorticoid treatment were examined by flow cytometer. The levels of CD4+CD25+ FOXP3+ Treg cells were higher in asthmatic children who had been receiving inhaled glucocorticoids, when compared to the control group and to the patients' levels before treatment ($p<0.05$). The present study suggests that at least one of the anti-inflammatory effects of inhaled glucocorticoids in asthma depends upon induction of Treg cells.

Key words: asthma, children, nTreg cells, glucocorticoid.

Allergic asthma is a complex and heterogeneous disease characterized by chronic inflammation of the bronchial mucosa and airway hyperresponsiveness (AHR). A majority of patients with asthma have an atopic background. The Th2 cell-derived cytokines interleukin (IL)-4, IL-5, and IL-13 play a central role in asthma and allergy[1-3]. The identification of transcription factors controlling Th1 and Th2 development further support the Th2 hypothesis since GATA3 is over-expressed and T-bet under- expressed in the asthmatic airway[4]. However, recent advances in both immunology and clinical phenotyping of asthma have raised the possibility that the other mechanisms may drive the pathology in some patients with asthma or coexist with Th2 type inflammation. Regulatory T (Treg) cells control the development of autoimmune disease, transplant rejection and allergic diseases and also play a key role in peripheral tolerance[5]. Although two distinct subsets of Tregs have been described to date, the best established are CD4+CD25+ Tregs, which emerge from the thymus: natural Tregs (nTreg). They constitute 5-10% of peripheral CD4+ T cells. These cells also express variable numbers of surface markers such as GITR, CTLA-4, CD152, neuropilin, and CD45 RO. However, the molecule that best defines the phenotype and function of nTregs is a transcription factor, namely the forkhead transcription factor 3 (FOXP3)[6,7]. Mutation of FOXP3 results in the depletion of CD4+CD25+ T cells and a syndrome called IPEX (immunodysregulation, polyendocrinopathy, enteropathy, X linked)[1,8]. The other category of Treg cells also derives from the thymus but acquires its suppressive activity in peripheral tissues: adaptive Tregs (aTreg)[9]. Two subsets of aTreg cells are defined according to cytokine expression: (i) Tr1 cells release IL-10 and (ii) Th3 cells release transforming growth factor (TGF)-β. Neither of these cells expresses FOXP3[10].

Corticosteroids bind to and activate the intracytoplasmic glucocorticoid receptor, which translocates into the nucleus and promotes anti-inflammatory genes. Inhaled glucocorticoids

are the most effective and widely used drugs for asthma. These drugs inhibit T cell activation and reduce the expression of Th2 type cytokines, which may contribute to their anti-inflammatory effects[11].

Thus, the aim of the present study was to obtain peripheral blood Treg cell levels among atopic asthmatic patients before and during inhaled steroid treatment and to identify the effect of these cells on the pathogenesis and treatment of asthma.

Material and Methods

Patient Characteristics

The study population consisted of 16 asthmatics and 20 age-matched healthy children. An informed consent was obtained from parents of both patients and controls.

The asthmatic group included 3 patients with moderate and 13 with mild asthma. All patients fulfilled the classification criteria according to the Global Initiative for Asthma (GINA) guidelines[12]. They were all symptomatic and were not receiving inhaled steroids or any other drugs at the beginning of the study. Skin prick test was conducted with a panel of common aeroallergen extracts in the presence of positive and negative controls on the forearm. All our patients were allergic to aeroallergens. Healthy children did not have asthma or other atopic or infectious diseases.

Ethylenediamine tetraacetic acid (EDTA)-anticoagulated blood was derived during the season in the morning for evaluation of the CD4+CD25+FOXP3+ T cell ratio, and subsequently, inhaled corticosteroids (fluticasone propionate in 5 of 16 patients, 250 μg/day or budesonide in 11 of 16 patients, 400

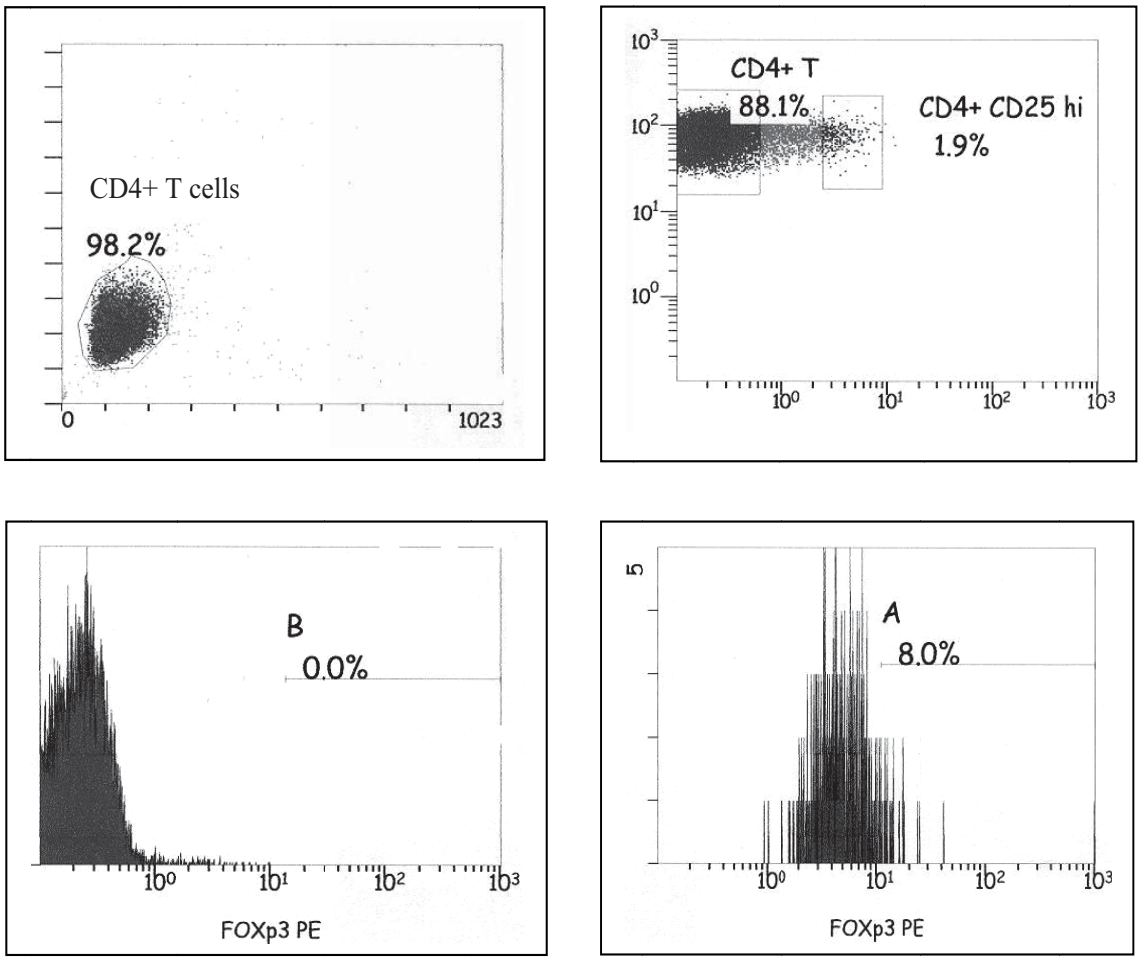

Figure 1: Example of quantitative value of FOXP3 level and fluorescence intensity (FI) in the CD4+CD25^{high} gate

μ/day) were started. No nasal steroids were given for comorbid allergic rhinitis during the study period. Approximately two months later (X±SD: 58.25 ± 19.49 - mean 56.0 days), the evaluation of the CD4+CD25+FOXP3+ T cell ratio was repeated when the symptoms were under control.

Phenotype Analysis

Three-color flow cytometry analysis was performed on peripheral whole blood collected in EDTA anticoagulant tubes. The blood cells were stained with antiCD4-PC5 (Immunotech, Marseille, France), antiCD25-FITC (Immunotech, Marseille, France) and intracellular anti-FOXP3-PE (eBioscience, San Diego, CA). Isotype-matched antibodies were used as negative controls.

Staining with mAb anti-FOXP3 (Antihuman FOXP3, eBioscience) was achieved according to the protocol recommended by the manufacturer (eBioscience) with IntraPrep Permeabilization Reagent (Immunotech, Marseille, France).

Three subsets of CD4+ T cells were defined according to CD25 staining: $CD25^-$, $CD25^{low}$ and $CD25^{high}$. Cells expressing $CD25^{high}$ were chosen and gated for the detection of FOXP3+ T cells (Fig. 1). Three-color cytometry was performed with Cytomics FC500 (Beckman Coulter) by using the CXP software version 2:1.

Statistical Analyses

The Statistical Package for the Social Sciences (SPSS) for Windows 11.5 was used for statistical analysis. Student t test was used to check age differences; Fisher's exact test was used for gender differences between the two groups. Mann-Whitney U test was used to test the difference in CD4+CD25+FOXP3+ T cell levels between the groups. Wilcoxon signed ranks test was performed to compare CD4+CD25+FOXP3+ T cell levels before and during treatment in the asthmatic group. The data are given as median (interquartile range) and 25^{th}-75^{th} percentiles. A p value of 0.05 or less was considered significant.

Results

There were no significant age or gender differences between control and asthmatic groups. Basic demographic features of patients and the control group are given in Table I.

The levels of CD4+CD25+ T cells expressing FOXP3 were found to be higher in asthmatic children who had been receiving inhaled glucocorticoids, when compared to the control group and to the patients' levels before treatment (Fig. 2).

Table I. Demographic Features of Asthmatic Patients and Controls

	Asthmatic children (n:16)	Controls (n:20)
Age (years)		
Mean	9.55 ± 3.72	10.63 ± 3.34
Range	4-16	4-16
Median	9.5	10.5
Gender		
Male/Female	13/3	13/7
Asthma severity		
Mild persistent	13	-
Moderate persistent	3	
Accompanying allergic rhinitis	15	-
Skin prick test positivity		
Grass pollen	10	All negative
House dust mite	3	
Tree pollen	1	
Alternaria	1	
Cat dander	1	
Treatment		
Inhaled fluticasone propionate	5	-
Inhaled budesonide	11	

Discussion

Asthma is the most frequent chronic disease of childhood; however, the pathogenetic mechanisms are not yet completely clarified. Th2 cytokines play a central role in asthma by secreting IL-4, IL-5 and IL-13[2]. Th1 cells might efficiently contribute to the effector phase in some allergic diseases depending on the stage of inflammation. Another subset of T cells with immunosuppressive function, Treg cells, inhibit both Th2 and Th1 responses[10]. There are two distinct phenotypes and mechanisms of action of Treg cells, including thymus-derived CD4+CD25+FOXP3+ naturally occurring (nTreg) and peripherally occurring adaptive Treg (Tr1 and TH3) cells. Adaptive Treg cells do not express FOXP3[13,14]. The role of Tregs in the pathogenesis of allergic diseases was not defined until recently. Treg may block the transition from the early activation stage to the differentiated Th2 state, limit airway infiltrates, and act to prevent inappropriate Th2 responses to environmental allergens.

There are few but important reports regarding the effect of glucocorticoids on CD4+CD5+FOXP3+ T cells in asthmatic patients. Karagiannidis et al.[15] reported that systemic glucocorticoids significantly increased the FOXP3 mRNA expression in adult patients with moderate and severe asthma, correlating with IL-10 mRNA expression. They showed that Tr1 cell induction depended on FOXP3 expression. The increase in CD4+CD5+FOXP3+ T cells with glucocorticoid treatment is in accordance with the results achieved in our study. It is strongly probable that glucocorticoids induce both adaptive and natural Treg cells simultaneously.

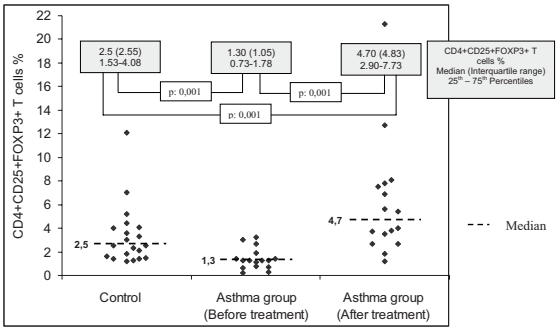

Figure 2: Data points and median levels of FOXP3 expressing T cells and CD4+CD25+FOXP3+ T cell levels of both control and asthmatic groups.

Recently, Provoost et al.[16] reported that FOXP3 protein expression within the CD4+CD25^{high} T cells was significantly lower in adult stable asthmatic patients compared with healthy adult control subjects. They also observed a tendency for increased FOXP3 protein expression within the CD4+CD25^{high} T cells in glucocorticoid-treated patients. In conclusion, they suggested that treatment with inhaled glucocorticoids in asthmatic patients might increase the FOXP3 protein expression. Because low levels of Tregs in asthmatic subjects increased with inhaled glucocorticoid treatment in our study, the results of Provoost's study strongly support our findings. The present study could also be a proof of their suggestion but it would be better to determine the FOXP3 mRNA levels.

This is the first longitudinal follow-up study in children with regard to CD4+CD5+FOXP3+ T cells and inhaled glucocorticoid treatment. The study reveals that inhaled glucocorticoids increase the CD4+CD5+FOXP3+ T cells in atopic asthmatic children and demonstrates that nTreg cells play important roles in the pathogenesis and treatment responses in asthma.

Glucocorticoids used in either inhaled or systemic form are the treatment of choice in asthma and allergic diseases because of their potent anti-inflammatory effects. Here, we have shown that inhaled glucocorticoids increase nTreg cell levels. This could be another anti-inflammatory mechanism of corticosteroids.

In conclusion, the results of this study suggest that one of the anti-inflammatory effects of inhaled corticosteroids is probably the induction of nTreg cells. Therefore, the manipulation of these cells is promising for the prevention/treatment of allergic disorders.

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